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Original Research

# Free radical scavenging activity of ethanolic extract of *Desmodium gangeticum*

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## Abstract

**Introduction:** The oxidation induced by reactive oxygen species can result in cell membrane disintegration, membrane protein damage, and DNA mutation. It can further initiate or propagate development of many diseases such as cancer, liver injury and cardiovascular disorders, whereby antioxidants with free radical scavenging activity play an important role in protecting damage by reactive oxygen species.

**Methods:** The present study assessed the contents of phenolics and flavonoids for their in vitro antioxidant activity such as 1,1-diphenyl-2-picrylhydrazyl, superoxide scavenging, reducing power, nitric oxide scavenging, 2,2 azino-bis(3-ethylbenzothiazoline-6-sulfonate), hydroxyl radical, ferric reducing antioxidant power, and chelating ability of ethanolic extract of *Desmodium gangeticum*.

**Results:** The total phenolic content of ethanolic extract of *D. gangeticum* measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent was  $16.2 \pm 0.7$  mg/mL. The flavonoid content of the plant sample calculated as catechol equivalent was  $10.5 \pm 0.5$  mg/mL. The antioxidative activity of the ethanolic extract of *D. gangeticum* was correlated with total phenolic content. Generally, the antioxidant activity of *D. gangeticum* ethanolic extract is comparable to a certain extent with that of butylated hydroxytoluene, mannitol, EDTA and ascorbic acid.

**Conclusion:** The results from this study indicate that the leaves of *D. gangeticum* possess antioxidant properties and could serve as free radical inhibitors, scavengers or primary antioxidants.

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**Keywords:** 2,2' azino-bis(3-ethylbenzothiazoline-6-sulfonate); *Desmodium gangeticum*; 1,1-diphenyl-2-picrylhydrazyl; Ferric reducing antioxidant power

## 1. Introduction

Free radicals produced in the body during normal metabolic functions or introduced from the environment are highly reactive species.<sup>1,2</sup> Free radicals contribute to more than 100 disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and acute AIDS. The body possesses defense mechanisms like enzymes and antioxidant nutrients, which arrest the damaging properties of reactive oxygen species.<sup>3,4</sup> The continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body, beyond its capacity to control them, and can cause irreversible oxidative damage.<sup>5</sup>

For several years, many researchers have been searching for powerful but nontoxic antioxidants from natural sources, especially edible or medicinal plants. Such natural antioxidants could prevent the formation of the above reactive-species-related disorders in humans, without the use of synthetic compounds, which may be carcinogenic and harmful to the liver and lungs.<sup>6</sup> The medicinal properties of plants have been investigated in the recent scientific world, because of their potent antioxidant activities, lack of side effects, and economic viability.<sup>7</sup>

*Desmodium gangeticum* (L) (Family Fabaceae) is a perennial shrub widely distributed in tropical and subtropical habitats, and particularly abundant in India.<sup>8</sup> This plant has been used in Ayurveda for the treatment of various diseases such as typhoid fever, urinary discharges, piles, inflammation, asthma, bronchitis, vomiting, dysentery and hemicranis.<sup>9</sup>

In the present study, the mode of antioxidant action in ethanolic extract of *D. gangeticum* was probed. *In vitro*

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methods of assessment were used to determine the scavenging activity of the extract on 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide scavenging, reducing power, nitric oxide scavenging, 2,2' azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), hydroxyl radical, ferric reducing antioxidant power (FRAP), and chelating ability.

## 2. Materials and methods

### 2.1. Sample collection and preparation

The leaves of *D. gangeticum* were collected from Kolli Hills in Namakkal District, Tamil Nadu, India. The samples were shade dried at room temperature and then ground to a fine powder in a mechanic grinder. The powdered material was then extracted using solvent ethanol in the ratio 1:10 using Soxhlet apparatus. After extracting all coloring material, the solvent was removed by evaporating in a water bath, which gave rise to a solid mass of the extract.

### 2.2. Chemicals

All chemicals used including the solvents were of analytical grade. Butylated hydroxytoluene, gallic acid, Folin–Ciocalteu reagent and ascorbic acid were purchased from Merck (Bangalore, India). All other chemicals and reagents used were of the highest commercially available purity. The solvents ethanol, methanol and sulfuric acid were purchased from Mercury (Tamil Nadu, India). DPPH free radical, ABTS, trichloroacetic acid, hydrogen peroxide, potassium ferric cyanide, sodium carbonate, sodium phosphate, ammonium molybdate, ascorbic acid and gallic acid were from Aldrich (Bangalore, India).

### 2.3. Estimation of total phenol and flavonoid content

Total phenolic content (TPC) was analyzed by the Folin–Ciocalteu colorimetric method using gallic acid as standard and expressed as mg/g gallic acid equivalent (GAE).<sup>10</sup> Flavonoid content was analyzed using catechol as a standard and this was expressed as mg/g catechol.<sup>11</sup>

### 2.4. Determination of antioxidant capacity

#### 2.4.1. DPPH radical quenching activity

Various concentrations of ethanolic extract of the sample (4.0 mL) were mixed with 1.0 mL methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM.<sup>12</sup> The mixture was shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as a control. The percentage of DPPH decolorization of the sample was calculated according to the equation:

$$\% \text{decolorization} = [1 - (\text{ABS}_{\text{sample}} / \text{ABS}_{\text{control}})] \times 100$$

IC<sub>50</sub> value (mg extract/mL) was the inhibitory concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison.

#### 2.4.2. Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazine methosulfate under aerobic condition<sup>13</sup>. The 3 mL reaction mixture contained 50 µL of 1 M NBT, 150 µL of 1 M nicotinamide adenine dinucleotide with or without sample and Tris buffer (0.02 M, pH 8.0). The reaction was started by adding 15 µL of 1 M phenazine methosulfate to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

#### 2.4.3. Reducing power

The reaction mixture contained 2.5 mL various concentrations of ethanolic extract of the sample, 2.5 mL 1% potassium ferric cyanide and 2.5 mL 0.2 M sodium phosphate buffer.<sup>14</sup> The control contained all the reagents except the sample. The mixture was incubated at 50°C for 20 minutes, and was terminated by the addition of 2.5 mL 10% (w/v) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. Five milliliters of the supernatant upper layer was mixed with 5.0 mL distilled water and 0.5 mL 0.1% ferric chloride, and absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicated increased reducing power of the sample. Ascorbic acid was used for comparison.

#### 2.4.4. Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside was measured.<sup>15</sup> Briefly, the reaction mixture (5.0 mL) containing sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25°C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion, which was assayed at 30-minute intervals by mixing 1.0 mL incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546 nm.

#### 2.4.5. FRAP

The stock solution of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl, 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O and 0.3M acetate buffer (pH 3.6) was prepared, as described previously.<sup>16</sup> The FRAP reagent contained 2.5 mL TPTZ solution, 2.5 mL ferric chloride solution and 25 mL acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 µL) was mixed with 90 µL water and 30 µL test ethanolic extract of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyl triazine (Fe<sup>3+</sup>-TPTZ) complex was reduced to ferrous (Fe<sup>2+</sup>) form. The absorption at 540 nm was recorded.

### 2.4.6. ABTS

Samples were diluted to 0.2–1.0 µg/mL. The reaction was initiated by the addition of 1.0 mL diluted ABTS to 10 µL of different concentrations of ethanolic extract of the sample or 10 µL methanol as a control.<sup>17</sup> The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation  $I = A_1 / A_0 \times 100$ , where  $A_0$  was the absorbance of the control reaction, and  $A_1$  was the absorbance of the test compound.

### 2.4.7. Hydroxyl radical activity

The reaction mixture (3.0 mL) contained 1.0 mL 1.5 mM FeSO<sub>4</sub>, 0.7 mL 6 mM hydrogen peroxide, 0.3 mL 20 mM sodium salicylate, and varying concentrations of the extract.<sup>18</sup> After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as Scavenging activity =  $[1 - (A_1 - A_2) / A_0] \times 100\%$ , where  $A_0$  was absorbance of the control (without extract),  $A_1$  was the absorbance in the presence of the extract, and  $A_2$  was the absorbance without sodium salicylate.

### 2.4.8. Iron chelating activity

The reaction mixture contained 1.0 mL of various concentrations of the extract, 0.1 mL 2 mM FeCl<sub>2</sub> and 3.7 mL methanol.<sup>19</sup> The control contained all the reaction reagents except for the sample. The reaction was initiated by addition of 2.0 mL 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability. The capacity to chelate Fe<sup>2+</sup> was calculated by percent chelation =  $[1 - (ABS_{\text{sample}} / ABS_{\text{control}})] \times 100$ .

## 3. Results

### 3.1. Total phenol and flavonoid content

The total phenolic content of ethanolic extract of *D. gangeticum* measured by Folin–Ciocalteu reagent in terms of gallic acid equivalent was 16.2 ± 0.7 mg/mL. The flavonoid content of the plant sample calculated as catechol equivalent was 10.5 ± 0.5 mg/mL. It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The results strongly suggest that the phenolics are important components of this plant, and some of the pharmacological effect could be attributed to the presence of this invaluable component.

### 3.2. DPPH

The reaction capability of DPPH radical was determined by the decrease in its absorbance at 515 nm induced by antioxidants. At 200–1000 µg, the antioxidant activities of ethanolic extract of *D. gangeticum* and the standard ascorbic acid were 25.12–54.14% and 27.03–63.19%, respectively. The extract exhibited concentration-dependent radical scavenging activity,

that is, the higher the concentration, the scavenging potential. The DPPH radical scavenging activity of ethanolic extract of *D. gangeticum* is shown in the Table 1, which clearly indicates the dose-dependent DPPH scavenging activity of *D. gangeticum*, with an IC<sub>50</sub> value of 820 µg, and ascorbic acid with an IC<sub>50</sub> value of 670 µg.

### 3.3. Superoxide scavenging

Superoxide radical reduced NBT to a blue-colored formation that was measured at 560 nm. At 200–1000 µg, the superoxide scavenging activity of ethanolic extract of *D. gangeticum* was 14.12–54.06% and that of the standard ascorbic acid was 17.11–54.06%. The superoxide scavenging activity of ethanolic extract of *D. gangeticum* and standard ascorbic acid is shown in Table 2. The ethanolic extract of *D. gangeticum* exhibited concentration-dependent radical scavenging activity, that is, percentage inhibition increased with sample concentration.

### 3.4. Reducing power

Ethanolic extract of *D. gangeticum* displayed considerable reducing power, primarily due to its effect as an electron donor, and thereby halting radical chain reactions by converting free radicals to more stable products. Increasing absorbance at 700 nm indicated an increase in reductive ability. Table 2 shows dose–response curves for the reducing power of the extract. The extracts showed good reducing power that was comparable with that of ascorbic acid. The antioxidant activity confirmed the medicinal importance of plants as naturally occurring antioxidants.

### 3.5. Nitric oxide scavenging activity

The minimum *in vitro* nitric oxide scavenging activity of the plant extract was 30.18% at 200 µg concentration, whereas the maximum *in vitro* activity was 67.3% at 1000 µg concentration. The percentage inhibition was increased with increasing concentration of the extract. However, activity of ascorbic acid was more pronounced than that of our extract. Fig. 1 clearly indicates the dose-dependent nitric oxide scavenging activity of *D. gangeticum*.

Table 1  
DPPH radical scavenging activity of ethanolic extract of *Desmodium gangeticum*.

Concentration (µg/mL)	DPPH (%)	
	<i>D. gangeticum</i>	Ascorbic acid
200	25.12 ± 0.44	27.03 ± 0.24
400	29.14 ± 0.36	32.70 ± 0.32
600	34.12 ± 0.48	48.11 ± 0.26
800	49.16 ± 0.24	52.08 ± 0.34
1000	54.14 ± 0.38	63.19 ± 0.46

Values are expressed as mean ± standard deviation (n = 3). DPPH = 1,1-diphenyl-2-picryl hydrazyl.

Table 2

Superoxide scavenging activity and reducing power by ethanolic extract of *Desmodium gangeticum*.

Concentration (mg/mL)	Superoxide scavenging (%)		Reducing power (absorbance)	
	<i>D. gangeticum</i>	Ascorbic acid	<i>D. gangeticum</i>	Ascorbic acid
200	14.12 ± 0.14	17.11 ± 0.22	0.28 ± 0.36	0.3 ± 0.19
400	25.14 ± 0.19	27.07 ± 0.26	0.47 ± 0.24	0.57 ± 0.24
600	36.28 ± 0.38	38.2 ± 0.29	0.62 ± 0.18	0.67 ± 0.25
800	47.14 ± 0.34	48.15 ± 0.32	0.72 ± 0.16	0.78 ± 0.29
1000	54.06 ± 0.42	57.7 ± 0.46	0.89 ± 0.18	1.01 ± 0.31

Values are expressed as mean ± standard deviation ( $n = 3$ ).

### 3.6. FRAP

The ferric reducing ability of the extract at 200–1000  $\mu\text{g}$  was in the range of 0.073–0.138 and that of the standard ascorbic acid was 0.084–0.146. The FRAP values for the extract were significantly lower than those of ascorbic acid. Fig. 2 shows that there was an increase in absorbance from 0.073 to 0.138.

### 3.7. ABTS

Proton radical scavenging is an important attribute of antioxidants. ABTS is a protonated radical that has a characteristic maximum at 734 nm, which decreases with the scavenging of proton radicals. Fig. 3 shows the ABTS scavenging ability of *D. gangeticum*. The scavenging effect of ABTS radical increased with concentration. The ethanolic extract of the leaves of *D. gangeticum* were fast and effective scavengers of the ABTS radical. The plant extract showed antioxidant activities, proving their capacity to scavenge ABTS.

### 3.8. Hydroxyl radical scavenging

The potential of an ethanolic extract of *D. gangeticum* to inhibit hydroxyl-radical-mediated deoxyribose damage was assessed at a concentration of 200–1000  $\mu\text{g/mL}$ . The sample exhibited minimum activity of 15.5% at 200  $\mu\text{g}$  and maximum activity of 56.12% at 1000  $\mu\text{g}$ , showing that the hydroxyl radical scavenging activity occurred in a dose-dependent manner (Fig. 4). The results indicate the scavenging potential of *D. gangeticum* against hydroxyl radicals.

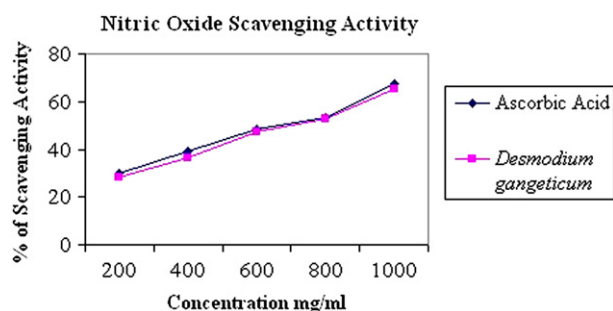


Fig. 1. Nitric oxide scavenging activity of ethanolic extract of *Desmodium gangeticum* and ascorbic acid. Values are expressed as mean ± standard deviation ( $n = 3$ ) indicate triplicate measurement.

### 3.9. Chelating ability

The ethanolic extract of *D. gangeticum* was evaluated for its chelating ability (Fig. 5). The absorbance of  $\text{Fe}^{2+}$ –ferrozine complex was decreased dose-dependently, that is, the activity was increased as concentration increased from 21.12% to 73.99%. EDTA showed strong activity. The minimum *in vitro* chelating ability of the plant extract was 21.12% at 200  $\mu\text{g}$  concentration and the maximum ability was 73.9% at 1000  $\mu\text{g}$  concentration.

### 3.10. Correlation between total phenolic content and total antioxidant activity

There was a correlation between total phenolic content and total antioxidant activity. The correlation co-efficient ( $R^2$ ; Fig. 6) of 0.9508 indicated a positive relationship between the total phenolics and total antioxidant activity. The strong correlation between antioxidant capacity and total phenolic content showed that phenolic compounds largely contribute to the antioxidant activities of the plant, and therefore could play an important role in the beneficial effects of the plant.

## 4. Discussion

### 4.1. Total phenol and flavonoid content

Polyphenols are the major plant compounds with antioxidant activity. Phenolic compounds are a class of antioxidants that act as free radical terminators.<sup>20</sup> The total phenolic content in plants usually correlates highly with the free-

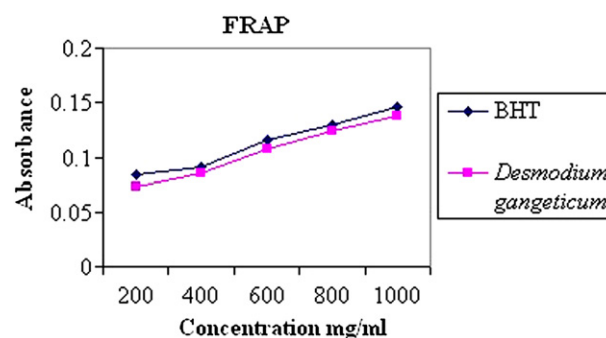


Fig. 2. Chelating ability of ethanolic extract of *Desmodium gangeticum* and EDTA. Values are expressed as mean ± standard deviation ( $n = 3$ ) indicate triplicate measurement.



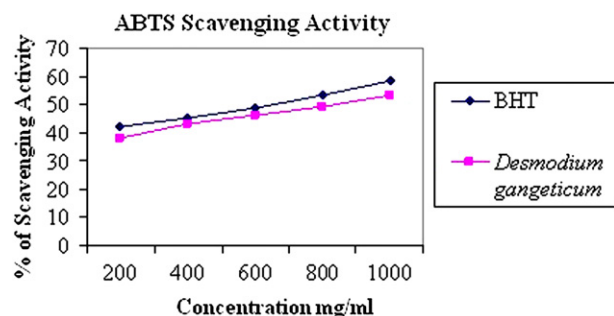


Fig. 3. Ferric reducing antioxidant power of ethanolic extract of *Desmodium gangeticum* and Butylated hydroxytoluene. Values are expressed as mean  $\pm$  standard deviation ( $n = 3$ ) indicate triplicate measurement.

radical-scavenging activity.<sup>21</sup> Compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for the antioxidant effect in plants.<sup>22</sup>

#### 4.2. DPPH

The free radical scavenging activity of ethanolic extract of *D. gangeticum* and also that of ascorbic acid was evaluated through its ability to quench the synthetic DPPH radical. There are many methods for evaluating the antioxidant activity of both natural and artificial compounds. The DPPH assay constitutes a rapid and low cost method that has frequently been used for evaluation of the antioxidative potential of various natural products.<sup>23</sup> Therefore, in the present study, *D. gangeticum* was screened for its possible antioxidant and radical scavenging activity by DPPH.

The radical scavenging reaction of ascorbic acid with DPPH was essentially instantaneous; the reaction of DPPH with *D. gangeticum* was also fast but slower compared to that with ascorbic acid. It is usually noticeable as discoloration of ethanolic extract of plant samples from purple to yellow; hence, DPPH is widely used to evaluate the free radical scavenging capacity of antioxidants.<sup>24</sup>

#### 4.3. Superoxide scavenging

Superoxides are produced from molecular oxygen by oxidative enzymes as well as via nonenzymatic reactions such

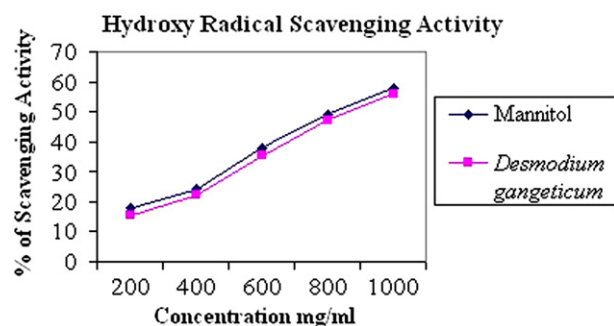


Fig. 4. ABTS scavenging activity of ethanolic extract of *Desmodium gangeticum* and Butylated hydroxytoluene (BHT). Values are expressed as mean  $\pm$  SD ( $n = 3$ ) indicate triplicate measurement.

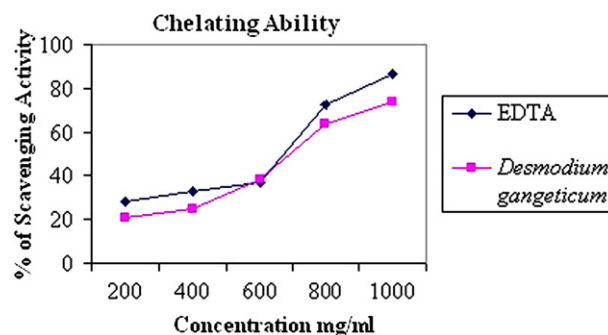


Fig. 5. Hydroxyl radical scavenging activity of ethanolic extract of *Desmodium gangeticum* and mannitol. Values are expressed as mean  $\pm$  standard deviation ( $n = 3$ ) indicate triplicate measurement.

as auto-oxidation by catecholamines.<sup>25</sup> Superoxide anions play an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, protein and DNA.<sup>26,27</sup> The superoxide scavenging activity of *D. gangeticum* was investigated, because the extract has the potential to scavenge superoxide anions. Fig. 2 clearly indicates that *D. gangeticum* is a potent scavenger of superoxide radicals.

#### 4.4. Reducing power

The reducing power of a compound acts as an indicator of its potential antioxidant activity.<sup>28</sup> The reducing properties are generally associated with the presence of reductones, which exhibit antioxidant activity by breaking the chain reactions by donating hydrogen atoms. Reductones also react with certain precursors of peroxide, thus preventing formation of the latter.<sup>29</sup> We showed that *D. gangeticum* had comparable reducing power towards ascorbic acid.

#### 4.5. Nitric oxide scavenging activity

Nitric oxide is a free radical produced in mammalian cells involved in regulation of various physiological processes. However, excess production of nitric oxide is associated with several diseases.<sup>30</sup> The development of substances to prevent the overproduction of nitric oxide has become a new research target for treating chronic inflammatory diseases.<sup>31,32</sup> In the

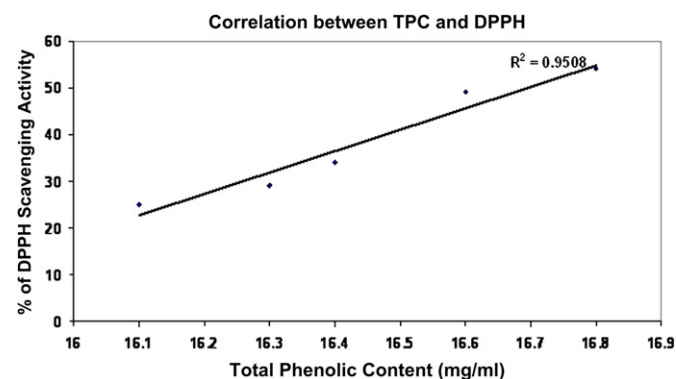


Fig. 6. Correlation between total phenolic content and total antioxidant activity of ethanolic extract of *Desmodium gangeticum*.

present study, the ethanolic extract of *D. gangeticum* was checked for its inhibitory effect on nitric oxide production. Nitric oxide radical generation from sodium nitroprusside at physiological pH was inhibited by *D. gangeticum*.

#### 4.6. FRAP

In FRAP, ferric-ferric cyanide complex is reduced to the ferrous form depending on the presence of antioxidants.<sup>33</sup> The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity; higher absorbance indicates a higher ferric reducing power.<sup>34</sup> We showed that *D. gangeticum* had comparable ferric reducing power to that of synthetic antioxidants.

#### 4.7. ABTS

The ABTS radical cation decolorization assay can measure the relative antioxidant ability to scavenge the radical ABTS as compared with BHT, and is an excellent tool for determining the antioxidant capacity of hydrogen-donating antioxidants. The blue and green ABTS radical cation was generated prior to adding antioxidant containing samples prevents interference, which stable absorbance was achieved, by adding the ethanolic extract of *Desmodium gangeticum* and the scavenging ability measured in terms of discolorization at 734 nm.<sup>35</sup>

#### 4.8. Hydroxyl radical scavenging

Hydroxyl radicals are highly potent oxidants, which can react with biomolecules in living cells and cause severe damage.<sup>36</sup> In the present study, administration of leaf extract to the reaction mixture significantly inhibited the hydroxyl radical activity, with a maximum inhibition of 58.06% and 56.12% being observed with the standard and *D. gangeticum*, respectively.

#### 4.9. Chelating ability

In the present study, there was overwhelming evidence that the ethanolic extract of *D. gangeticum* had high chelating activity *in vitro*. The presence of chelating agent complex formation is disrupted, which reduces the red color of the complex. This measurement of color reduction therefore allows estimation of the chelating ability of the coexisting chelator. The transition metal ion,  $\text{Fe}^{2+}$ , possesses the ability to move single electrons, which allows the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals.<sup>37</sup>

### 5. Conclusion

As our knowledge of the mechanisms of human diseases has increased, particularly metabolic diseases such as diabetes, liver disease and hypertension, the role played by highly reactive oxygen species such as free radicals has become increasingly relevant. Research on medicinal plants for natural

antioxidants is also increasing. The present study indicates that the leaves of *D. gangeticum* possess antioxidant properties and could serve as free radical inhibitors or scavengers, or act as primary antioxidants. A significant relationship between the antioxidant capacity and total phenolic content was found. With this kind of investigation it would be easier to treat and prevent the human damages occurring due to the free radical. Therefore, further research is needed for the isolation and identification of the active components in the extracts.

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